Docket No.
2531-1-001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS	:	Michael Meyrick Burrell et al.
SERIAL NO.	:	09/383,579
FILED	:	August 25, 1999
FOR	:	MODIFICATION OF PLANT FIBRES

Certificate of Mailing Under 37 CFR 1.8

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to COMMISSIONER FOR PATENTS, P.O. BOX 1450, ALEXANDRIA, VA 22313-1450 on August 24, 2004.

Carolyn Di Meglio
(Name of Depositor)

Carolyn Di Meglio 8/24/04
(Signature and Date)

PETITION FOR GRANT OF PRIORITY UNDER 35 USC 119

COMMISSIONER FOR PATENTS
P.O. BOX 1450
ALEXANDRIA, VA 22313-1450

Dear Sir:

Applicant hereby petitions for grant of priority of the present Application on the basis of the following prior filed foreign Application:

COUNTRY	SERIAL NO.	FILING DATE
GREAT BRITAIN	**9818808.9**	**AUGUST 29, 1998**

To perfect Applicant's claim to priority, a certified copy of the above listed prior filed Application is enclosed.

Acknowledgment of Applicant's perfection of claim to priority is accordingly requested.

Respectfully submitted,

David A. Jackson
Attorney for Applicant
Registration No. 26,742

KLAUBER & JACKSON
411 Hackensack Avenue
Hackensack, NJ 07601
(201)487-5800

THIS PAGE BLANK (USPTO)

The Patent Office

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

CERTIFIED COPY OF PRIORITY DOCUMENT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed R. Mahoney

Dated 14 April 2004

CERTIFIED COPY OF PRIORITY DOCUMENT

Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
J
29 AUG 1998
RECEIVED BY POST

The Patent Office

THE PATENT OFFICE
J
~~27 AUG 1998~~
~~RULE 97~~
RECEIVED BY POST

1/77

01SEP98 E386679-1 D01886
P01/7700 25.00 - 9818808.9

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

---

1. Your reference — RD-ATC-19

2. Patent application number *(The Patent Office will fill in this part)* — **9818808.9** — 29 AUG 1998

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

   Advanced Technologies (Cambridge) Limited
   Millbank
   Knowle Green
   Staines
   Middlesex TW18 1DY
   England

   Patents ADP number *(if you know it)* — 04458832002

   If the applicant is a corporate body, give the country/state of its incorporation — England & Wales

4. Title of the invention — **MODIFICATION OF PLANT FIBRES**

5. Name of your agent *(if you have one)*

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

   Mrs. M.R.Walford / K.J.H. MacLean
   Patents Department
   British American Tobacco (Investments) Limited
   R&D Centre
   Regents Park Road
   Southampton SO15 8TL
   England

   Patents ADP number *(if you know it)* — 07411630001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
   a) any applicant named in part 3 is not an inventor, or
   b) there is an inventor who is not named as an applicant, or
   c) any named applicant is a corporate body.
   See note (d))

   YES

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	30 pages x 2
Claim(s)	
Abstract	
Drawing(s)	INFORMAL 1 sheets x 2

10. If you are also filing any of the following, state how many against each item.

Priority documents	-
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Margot Walford Date 28.8.98

Margot Ruth WALFORD

12. Name and daytime telephone number of person to contact in the United Kingdom

Mrs. M.R.Walford 01703 793730

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

Modification of Plant Fibres

This invention relates to the modification of the morphology of plant fibre cells. The invention is exemplified by methods of using genetic constructs for the modification of, in particular, but not exclusively, *Eucalyptus* fibres, for example.

The primary product of the forestry industry is considered to be wood, although more fundamentally it could be defined as fibre. The industry supplies a wide range of feedstocks to the solid wood and pulp/paper industries who produce a multiplicity of products. The forester must therefore seek to cater for the competing needs of these industries, and even within the individual industries, there is a range of different requirements. For example, different paper grades require different qualities in the starting material.

Forestry-based operations depend upon a balance between the capability of the forester to supply the processor with fibre having specific properties, and the ability of the processor to modify his process and so accommodate the available feedstock. The design and operation of processing plants are influenced by the wood (fibre) properties of the feedstock.

Notwithstanding these specific demands, fibre uniformity and strength are common requirements for most industrial uses,

and hence the fibre supplied by the forester must be capable of delivering these properties to the processor.

In pulp manufacture, for example, strength characteristics are determined in part by fibre length. Increased fibre length leads to the production of paper with increased strength. Bond strength is attributed to contact between the fibres and the adhesion capabilities of the surfaces, which are dependent upon fibre length, perimeter and coarseness. Also, during the manufacturing process, increased fibre length increases the strength of wet webs enabling easier handling (Seth, 1995).

However, long fibres are not desirable for all applications. In some cases, shorter fibres are preferable, such as in the production of smooth-surfaced papers.

Fibre properties differ between species, and consequently particular species have been limited historically to particular applications. Fibres from hardwood species are generally much shorter than those from softwoods. This results in the production of pulp and paper with desirable surface characteristics such as smoothness and brightness, but with low strength characteristics. In practice, where a single species providing fibre with an appropriate combination of characteristics has not been available, the mixing of long and short fibres from different species is used. If a single source were available, possessing the desirable characteristics plus optimal fibre length, this would be of great benefit to the processor. Some common species and their fibre lengths are exemplified in Table 1 below.

Table 1

Fibre Lengths of Various Tree Species

Species	Fibre Length (mm)
Loblolly Pine	3.5 - 4.5
Western Hemlock, Western Spruce	2.5 - 4.2
Southern Hardwood	1.2 - 1.4
Northern Hardwood	1.0 - 1.2
Eucalyptus	0.8 - 1.0
White Oak	0.59
Sweetgum	0.48
Aspen	0.35

Eucalyptus trees represent the largest sources of fibres used globally in the paper industry (Bamber 1985; Ranatunga, 1964), and world-wide, there are an estimated ten to fifteen million hectares of land planted with *Eucalyptus* (Verhaegen and Plomion 1996). The major advantage of Eucalypts is their very high growth rates and ability to grow in a wide range of conditions, both tropical and temperate.

However, *Eucalyptus* fibres are significantly shorter than those from other, once more popular, sources of fibre such as pine. Thus papers that are made from *Eucalyptus* pulp are often weak and usually require reinforcement with longer fibres from other sources increasing the production costs. If trees could be produced with longer fibres, this would be a considerable advantage to the paper industry, increasing the quality of the raw materials for pulp and paper synthesis.

Through tree breeding it is possible to achieve some modification of fibre characteristics. For example, interspecific triploid hybrids of poplar have been developed which have longer fibres than the parental species.

Genetic variation in fibre properties is also evident within species. Fibre characteristics are controlled by a complex set of genetic factors and are not easily amenable to classical breeding methods. Therefore, existing genetic variation has not been exploited significantly in tree breeding programmes. Whilst knowledge is now being accumulated on the heritability of wood properties, previously these were not often considered as important as growth characteristics and were sometimes sacrificed in pursuit of the latter. In some instances, growth rate is negatively correlated with fibre characteristics, though this does not always hold true (e.g. in Eucalypts), and breeding efforts are now being made to capture the benefits of both.

In many cases fibre properties are sufficient for the end product, and improvement is considered unnecessary. For example, increasing fibre length beyond 2mm causes little increase in tear strength or tensile strength, and many softwood fibres are commonly around 3mm long, i.e. greater than the minimum for desired strength. However, fibres in juvenile wood tend to be shorter and there is an increased usage of juvenile material through a reduction in rotation times. Hence, there is scope for improvement even in those species which commonly yield long fibres.

From the perspective of the pulp and paper industry, fibres are specific types of plant cell walls that have been subjected to a range of treatments to remove all contents and most non-cellulosic wall components (Stewart *et al*, 1994). In woody plants the fibres are made up of dead cell wall material. In order to produce longer fibres it is necessary to have longer living cells during growth, before fibre formation.

The cell wall can be envisaged as a complex network of cellulose microfibrils linked together by noncovalent interactions with matrix polymers (Carpita and Gibeaut), 1993). The microfibrils are coated by a mixture of hemicelluloses which form extensive hydrogen-bonded interactions with the surface of the microfibrils. Coextensive with this is another network formed from various pectins which are held together largely by ionic linkages (McQueen-Mason, 1995).

To allow cells to grow and enlarge the wall components must loosen to enable slippage of the polysaccharides and proteins within the matrix (Cosgrove, 1993). Extension of the cell is then driven by the internal turgour pressure of the cell, which is considerable. The degree of extension during cell growth is controlled by the mechanical properties of the cell wall, which result from their composition and from the orientation of wall fibrils and structural polymers.

The control of cell wall extension is closely regulated by the plant to facilitate growth control and morphogenesis. The ultimate agents of control are enzymes located in the wall

itself. If plants express cell wall "loosening" enzymes in their walls, then it seems likely that these enzymes can regulate cell growth. Altered levels of expression can thereby cause increased or reduced cell growth and fibre length. Changes in cell wall texture may also be produced.

One class of cell wall proteins are the Expansins. Expansins induce the extension of plant walls, and at present are the only proteins reported with demonstrated wall-loosening activity. Expansins were first isolated from cucumber hypocotyl cell walls by McQueen-Mason *et al* (1992) and characterised by their ability to catalyse wall loosening in an *in vitro* rheological assay.

The mode of action of expansins is believed to be by weakening the noncovalent bonding between the cellulose and hemi-cellulose, with the result that the polymers slide relative to one another in the cell wall (Cosgrove 1996). The precise biochemical action of expansins is unclear, although it is known that their effects are not due to exoglycanase or xylogucan endotransglycosylase activity (McQueen-Mason *et al*, 1992, McQueen-Mason & Cosgrove, 1993). Expansins appear to disrupt hydrogen bonding between cellulose microfibrils and hemicelluloses. The process enables wall loosening without any degradation of the polymers or an overall weakening of wall structure during expansion. Consistent with this mechanism, expansins have been shown to weaken cellulosic paper, which derives its mechanical strength from hydrogen bonding between cellulose fibres (McQueen-Mason and Cosgrove, 1994).

Expansins are able to restore the ability of isolated cell walls to extend in a pH dependent manner (McQueen-Mason and Cosgrove, 1995) and may be responsible for the phenomenon of "acid growth" in plants (Shcherban *et al*, 1995). Expansin proteins have been characterised in cucumber hypocotyls (McQueen-Mason *et al*, 1992), oat coleoptiles (Li *et al*, 1993), expanding tomato leaves (Keller and Cosgrove, 1995) and rice internodes (Cho and Kende, 1997).

Expansin cDNAs have been isolated and characterised from a number of plants and it is now evident that expansins exist as a multi-gene family showing a high level of conservation between species. cDNAs with high degrees of homology have been identified from collections of anonymous Expression Sequence Tag (EST) cDNAs from *Arabidopsis* and rice. These EST cDNAs exhibit a high degree of homology at the level of protein sequence (60-87%) indicating that expansin structure is highly conserved (Shcherban *et al* 1995). Expansins show no sequence similarity to other known enzymes, although they do have sequence similarities to some pollen allergens (Shcherban *et al*, 1995). Recently Cosgrove *et al* (1997) have shown that pollen allergens from maize also possess considerable expansin activity.

If plants can be modified to over-express expansins in their walls, then it seems likely that these plants will exhibit a marked increase in cell extension or growth. Conversely, a reduction in the expression of expansins should lead to a reduction in cell growth.

One approach to modifying the expression of expansins is via the introduction of recombinant DNA sequences into the plant genome. Several methods can be used to introduce foreign DNA into plant cells (see review by Weising *et al*, 1988; Miki and Iyer, 1990 and Walden 1994). *Agrobacterium tumefaciens*-mediated gene transfer is probably the most widely used and versatile of these methods (Walden, 1994).

Genetic modification experiments directed towards changing the wood and paper quality of trees has been investigated by other workers, particularly focusing on the lignin pathway in cells and lignin content in the final paper product (Hawkins and Boudet, 1994; Grima-Pettenati, *et al*, 1993; Poeydomenge *et al*, 1993; Boudet *et al*, 1995 and Hibino *et al*, 1994). The aim of the present invention differs in that it seeks to provide a means of controlling fibre growth and cell wall morphology.

An object of the present invention is to provide a method whereby trees can be modified to produce fibres of a desired length for specific applications. This will enable the forester to control the quality of his product. In addition it will enable the forester to produce a wide range of fibre types from a single or small number of species which can be selected as being ideally suited for cultivation in that particular site. This will result in both the economy of employing a single uniform silvicultural regime, and the flexibility of producing which ever type of fibre is required at a particular time.

The invention also provides a means of producing fibre of specific type from trees at particular periods in their growth cycle. For example, the production of long fibres from juvenile trees can be achieved, thereby accelerating the time to harvest of the crop.

This is achieved by firstly isolating and characterising expansin gene sequences from heterologous and homologous species and then reintroducing these genes into trees so as to alter expansin levels in the transgenic trees using over-expression, co-suppression and anti-sense knockout strategies. This will lead to the cultivation of trees more suitable for paper production.

The present invention provides a nucleic acid coding sequence encoding a gene capable of modifying the extension of fibre cell walls, the nucleic acid coding sequence being one or more of SEQ.ID. Nos. 1-6 hereof.

The present invention also provides a method of transforming trees to modify the fibre characteristics in trees, the method comprising stably incorporating into the plant genome a chimaeric gene comprising a promoter, a nucleic acid coding sequence encoding a gene capable of modifying the extension of fibre cell walls, and a terminator, and regenerating a plant having an altered genome.

The present invention also provides trees having therein a chimaeric gene comprising a promoter, a nucleic acid coding sequence capable of modifying the extension of fibre cell walls and a terminator.

Constructs and chimaeric genes having the DNA structural features described above are also aspects of the invention.

Plant cells containing chimaeric genes comprising a nucleic acid coding sequence capable of modifying the extension of fibre cell walls are also an aspect of this invention, as is the seed of the transformed plant containing chimaeric genes according to the invention.

The chimaeric gene may comprise the nucleic acid coding sequence as it exists in the genome, complete with endogenous promoter, terminator, introns and other regulatory sequences, or the nucleic acid coding sequence, with or without introns, may be combined with a heterologous promoter, terminator and/or other regulatory sequences.

The promoter may be a constitutive promoter, such as the cauliflower mosaic virus 35S gene or nopaline synthase promoter, a tissue specific promoter, such as *rol*C or an inducible promoter, such as AlcR/AlcS. Other suitable promoters will be known to those skilled in the art.

The nucleic acid sequence, or parts thereof, may be arranged in the normal reading frame direction, i.e. sense, or in the reverse reading frame direction, i.e. antisense. Up or down regulation of the activity of the expansin or expansin gene using sense, antisense or co-suppression technology may be used to achieve alteration in the length of fibre cell walls.

Preferably the nucleic acid sequence encodes one or more of the class of proteins known as expansins. The nucleic acid sequence may advantageously be one or more of SEQ. ID. Nos. 1-

6 hereof. Alternatively, the nucleic acid sequence may be a sequence which has sufficient homology to hybridise to any one of SEQ. ID. Nos. 1-6 under medium stringency conditions (washing at 2x SSC at 65°C).

Preferably the nucleic acid sequence is an mRNA or cDNA sequence, although it may be genomic DNA.

Preferably the nucleic acid sequence is derived from *Eucalyptus* or cucumber.

Trees which may suitably be transformed using the inventive method include Eucalypts, Aspen, pine, larch.

The nucleic acid sequence may be introduced by any of the known genetic transformation techniques such as *Agrobacterium tumefaciens* mediated transformation, *Agrobacterium* rhizogenes mediated transformation, biolistics, electroporation, chemical poration, microinjection or silicon-fibre transformation, for example.

In order that the invention may be easily understood and readily carried into effect, reference will now be made, by way of example, to the following Figures, in which:-

Figure 1a is a diagrammatic representation of the coding sequence for cucumber Ex29 cloned between the cauliflower mosaic virus 35S promoter and nos terminator in the vector pDE326;

Figure 1b is a diagrammatic representation showing the insert from Figure 1 between the EcoR I and Hind *III* restriction sites introduced into a modified Ti plasmid pDE 1001 to produce pDE/EXP29, and

Figure 1c is a diagrammatic representation showing the insert from Figure 1 between the EcoR I and Hind *III* restriction sites introduced into a modified Ti plasmid p35GUSINT to produce pATC/EXP29;

MATERIALS AND METHODS

RNA Extraction and mRNA Isolation:

RNA extraction from cucumber hypocotyls. Seeds of cucumber (*Cucumis sativus* L., cv Burpee pickler, from A.W. Burpee, Westminster, Penn, USA) were sown on water-soaked capillary matting (Fordingbridge Growers Supplies, Arundel, W. Sussex, UK) in plastic trays (35cm x 25cm x 6cm) and germinated in the dark at 27°C. After 4 days the etiolated seedlings were harvested under green light by excising the upper 20mm of the hypocotyl into liquid nitrogen and grinding to a fine powder in a pestle and mortar that had previously been chilled at -80°C. Total RNA was extracted in a hot phenol/lithium chloride buffer according to the procedure of Verwoerd *et al* (1989).

RNA extraction from *Eucalyptus grandis*. *E.grandis* seeds were sown on trays (35cm x 25cm x 6cm) of Levington's F2 compost (Levington Horticulture Ltd., Ipswich, Suffolk, UK) and germinated in a greenhouse (18-24°C, at a light intensity of approximately 10,000 lux, and 16 hours of daylight). After 8 weeks the seedlings were transferred to individual pots, and then repotted as necessary (approximately every 6-7 weeks). Growing stem tissue was harvested from the last 40-50mm of branch tips into liquid nitrogen. Immature leaves, usually

the youngest two from growing branch tips, were also harvested directly into liquid nitrogen; roots were washed in several bowls of tap water, rinsed with distilled water and then growing tips were excised into liquid nitrogen. RNA was extracted as described by Pawlowski *et al* (1994) using a protocol especially modified for the extraction of RNA from plants containing high levels of phenolic compounds.

Poly(A^+) mRNA isolation from total RNA extracted from *E.grandis* stem tissue. Poly(A^+) mRNA was isolated from total RNA using either push (Stratagene, Cambridge, UK) or spin oligo(dt) columns (Clontech Laboratories, Inc. CA., USA) and following the supplier's instructions and recommendations.

RT-PCR and Sequencing

The nucleic acid sequence of expansins show a considerable extent of divergence. However two regions with a reasonable degree of consensus were identified and used to synthesise two oligonucleotide primers of low complexity.

Total RNA was extracted from young stem tissue and Poly(A^+) mRNA isolated using oligo(dt) columns as described above. 1μg of mRNA was used in a PCR experiment (50°C annealing temperature, 30 cycles, hot start) with the two expansin consensus primers (see Table 2) and Taq DNA polymerase (Promega UK Ltd.).

Table 2

Sequence of Consensus Expansin Primers

	Sequence (5'-3')
P.1 (SEQ. ID. No. 7)	ATGGIGGIGCNTGYGGNTA
P.2 (SEQ. ID. No. 8)	TGCCARTTYTGNCCCCARTT
Key: Y=C or T, N=A or G or C or T, R=A or G, I=Inosine	

cDNA Library Consruction

For first strand cDNA synthesis 1μg of mRNA was used in a reaction with 0.15μg OG1 oligo dt primers and AMV Reverse Transcriptase (9 units/μl, Promega UK Ltd., Southampton, UK).

The library was constructed in the Lambda ZAP II vector (Stratagene, Cambridge, UK), following the supplier's instructions.

Northern Analysis

Total RNA was isolated from the stem, leaves and roots of *E. grandis* as described above. 6μg of RNA in 20μl DEPC H_2O was denatured in a equal volume of denaturing solution (50% formamide, 2x TBE) and run on a standard 1.5% agarose gel at 75 volts for 200 min. RNA from the gel was transferred onto "Zeta-Probe" GT Genomic Tested Blotting Membranes (Biorad Laboratories, California, USA) by capillary transfer. Partial *E.grandis* expansin sequences generated by RT-PCR from stem mRNA (as described above) were used for 32P-random prime labelling and hybridised to the transferred RNA following the membrane supplier's recommended methods (Biorad Laboratories).

Vector Construction

The coding sequence for cucumber Ex29 (GenBank Accession No. U30382; known as Cs-EXP1, and Shcherban *et al* 1995) was generated by RT-PCR and cloned between the Cauliflower Mosaic Virus 35S promoter and *nos* terminator (see Figure 1a) into pDE326, a vector kindly donated by Dr. Jürgen Denecke of York University. After insertion of the Ex29 expansin sequence the inserts were sequenced to check for correct in frame insertion by sequencing using a primer located within the 35S promoter region.

Inserts containing the 35S promoter, Ex29 sequence and *nos* terminator were cut between the EcoRI and Hind*III* restriction sites and inserted into modified Ti plasmids to produce transformation constructs. Two modified Ti plasmids were used: pDE1001 (Denecke *et al*, 1992 or Shcherban *et al* 1995) provided by Dr. Jürgen Denecke and p35GUSINT (Vancanneyt *et al*, 1990). The plasmids containing the insert were referred to as pDE/EXP29 (pDE1001 + Ex29) (see Figure 1b) and pATC/EXP29 (p35GUSINT + Ex29) (See Figure 1c), acknowledging the source of the plasmids. Plasmids were transferred into *E.coli* by standard procedures; *E.coli* strains were grown on LB plates (incubated at 37°C and stored at 4°C) or in LB medium with the appropriate antibiotic for positive selection.

Two strains of *Agrobacterium tumefaciens* were used. A C58 strain (C58C1(pGV2260) Deblaere, R. *et al* 1985) kindly donated by Dr. Jürgen Denecke, and EHA105 deposited by Advanced Technologies (Cambridge) Limited of 210 Cambridge Science Park, Cambridge CB4 4WA, under the Budapest Treaty on

the International Recognition of the Deposit of Micro-organisms for the purposes of Patent Procedure at the National Collection of Industrial and Marine Bacteria (NCIMB), 23 St. Machar Street, Aberdeen, Scotland on 25 August 1998 under Accession No. NCIMB 40968. *Agrobacterium* were grown on LB plates (incubated at 27°C and stored at 4°C) or in LB medium with the appropriate antibiotic for positive selection. The constructs were introduced into *Agrobacterium* via direct DNA transformation or by tri-parental mating using the *E.coli* mobilisation function strain HB101 (pRK2013) (Figurski and Helinski 1979)

Plant Transformation

Young leaves were dissected under sterile conditions, from approximately 4 week old *E.grandis* cultures grown in Magenta boxes (7cm x 7cm x 13cm) on LS media at 25°C, in a growth room in our tissue culture laboratory and used for *Agrobacterium*-mediated infection (Horsch, Fry, Hoffman, Eichholtz, Rogers, and Fraley 1985). Inoculated tissue was left to co-cultivate for 4d on LS media (plus 20g/l glucose, 0.7% agarose, 0.01mM Zeatin a 1μM NAA) in diffuse light in a growth room, conditions as before. Transformants were selected on 50mg/l kanamycin and 250mg/l claforan.

RESULTS

Isolation of Novel Expansin Sequences from *E.grandis* stem tissue

Using the methods described, transformed pTAG clones were isolated by blue-white colony selection on agar plates following the methods described by the supplier (R&D Systems). Twenty white ("positive") colonies were selected and sequenced. Of these, six were identified as containing sequences that had similarities with other known expansin sequences using a basic BLAST search provided by NCBI. The putative transcripts were all around 450 bps in size (determined by PCR and gel electrophoresis). PCR products were sequenced using a forward primer and the sequences identified as SEQ.ID. Nos. 1-6 were obtained.

Vector Construction and Plant Transformation

As described in the Methods two constructs for plant transformation were prepared and introduced into two strains of *Agrobacterium*, C58 and EHA 105 to produce C58 containing pDE + Ex29, C58 containing pATC + Ex29 and EHA105 containing pATC + Ex29. Each construct-containing strain was used to inoculate 400 leaves dissected from *E.grandis* tissue (on two separate occasions, each time inoculating 200 leaves).

The transformation experiments were repeated with a further 240 leaves, inoculated with EHA105 containing pATC + Ex29 to increase the amount of possible transformants obtainable.

From the original batch of inoculated tissue with EHA105, 25 plants were grown in the greenhouse and the properties of the shoots determined.

REFERENCES

Bamber RK (1985) The wood anatomy of Eucalypts and papermaking. *Appita* **38**:210-216

Boudet AM, Lapierre C + Grima-Pettenati J (1995) Biochemistry and molecular biology of lignification. New Phytol. Tansley rev. **80**, 129 203-236

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J **3(1)**: 1-30.

Cho H-T, Kende H (1997) Expansins in deepwater rice internodes. Plant Physiol **113** 1137-1143

Cho H-T, Kende H (1997) Expansins and internodal growth of deepwater rice. Plant Physiol **113** 1145-1151

Cosgrove DJ 1993 Wall extensibility - its nature, measurement and relationship to plant cell growth. New Phytol **124(1)**:1-23

Cosgrove DJ (1996) Plant cell enlargement and the action of expansins. *BioEssays* **18(7)**:533-540

Cosgrove DJ, Bedinger P, Durachro (1997) Group I allogens of grass pollen as cell wall loosening agents. Proc. Nat. Acad. Sci. USA 94 6559-6564

Deblaere, R. Bytebier, B. De Greve, H., DeBoaeck, F., Schell, J., Van Montagu, M. Leemans, J. (1985) Efficient octopine T-

plasmid vectors for *Agrobacterium*-mediated gene transfer to plants. Nucl. Acid. Res., 13, 4777-4788

Denecke J. Rycke RD, Botterman J (1992) Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. *The EMBO Journal* **11(6)**:2345-2355

Figurski D and Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Nat. Acad. Sci. USA, 76, 1648-1652

Grima-Pettenati J, Feuillet C, Goffner D, Borderies G, Boudet AM (1993) Molecular cloning and expression of a *Eucalyptus gunnii* clone encoding cinnamyl alcohol dehydrogenase. *Plant Mol Bio* **21**:1085-1095

Hawkins SW, Boudet AM (1994) Purification and characterisation of cinnamyl alcohol dehydogenase isoforms from the periderm of *Eucalyptus gunnii* Hook. *Plant Physiol* **104**:75-84

Herrera-Estrella I, De Block M, Messens E, Hernalsteens JP, Van Montagu M, Schell J (1983) Chimeric genes as dominant selectable markers in plant cells. *The EMBO Journal* **2**:987-995

Hibino T, Chen J-Q, Shibata D + Miguchi T (1994) Nucleotide sequences of a Eucalyptus botryoides gene encoding dinnamyl alcohol dehydrogen. Plant Physiol. 104 305-306

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* **227**: 1229-2123

Keller E, Cosgrove DJ (1995) Expansins in growing tomato leaves. *Plant J* **8**:795-802

Li Z-C, Durachko DM, Cosgrove DM (1993) An oat coleoptile wall protein that induces wall extension in vitro and that is antigenically related to a similar protein from cucumber hypocotyls. *Planta* **191**:349-356

McQueen-Mason S (1995) Expansins and cell wall expansion. *J. Exp. Bot.* **B46(292)**:1639-1650

McQueen-Mason S, Cosgrove DJ (1993) Cucumber expansins disrupt hydrogen-bonds between cellulose fibers in vitro. *Plant Physiol* **102(1)**: 122

McQueen-Mason S, Cosgrove DJ (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. *Proc Natl Acad Sci USA*91:6574-6578

McQueen-Mason S, Cosgrove DJ (1995) Expansin mode of action on cell walls; Analysis of wall hydolysis, stress relaxtion and Binding. *Plant Physiol* **107**:87-100

McQueen-Mason S, Durachko DM, Cosgrove DJ (1992) Two endogenous proteins that induce cell wall extension in plants. *Plant Cell* **4**:1425-1433

Miki BLA, Iyer VN (1990) Fundamentals of gene transfer in plants. In: Dennis DT, Turpin DH (eds) Plant Physiology, Biochemistry and Molecular Biology, 1st edn. Longman Scientific & Technical Publishers, UK

Pawlowski K, Kunze R, De Vries S, Bisseling T (1994) Isolation of total, poly(A) and polysomal RNA from plant tissues. Kluwer Academic Publishers, Belgium

Poeydomenge O, Boudet AM, Grima-Pettenati J (1994) A cDNA encoding S-adenosyl-L-methionin:caffeic acid 3-O-methyltransferase from *Eucalyptus*. *Plant Physiol* **105**:749-750

Ranatunga MS (1964) A study of the fibre lengths of *Eucalyptus grandis* grown in Ceylon. *Ceylon For* **6**:101-112

Shcherban TY, Shi J, Durachko DM, Guiltinan MJ, McQueen-Mason S, Shieh M, Cosgrove DJ (1995) Molecular cloning and sequence analysis of expansins - a highly conserved and multigene family of proteins that mediate cell wall extension in plants. *Proc. Natl Acad Sci USA* **92**:9245-9249

Seth RS(1995)The effect of fibre length and coarseness on the tensile strength of wet webs: a statistical geometry explanation. Tappi J 78 (3) 99-102

Stewart *et al* (1994)

Vanncanneyt, G, Schmidt, R., O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990)Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol. Gen. Genet. 220, 245-250

Verhaegen, D, Plomion C (1996) Genetic mapping in *Eucalyptus urophylla* using RAPD markers. *Genome* **39**:1051-1061

Verwoerd TC, Dekker BMM, Hoekema A (1989) A small scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. **17(6)**:2362

Walden R (1994) Cell Culture, Transformation and Gene Technology. In: Lea PJ, Leegood RC (eds) Plant Biochemistry and Molecular Biology, 1st edn. John Wiley & Sons Ltd., London

Weising K, Schell, J, Kahl G. (1988) Foreign genes in plants: transfer, structure, expression and applications. Ann. Rev. Genet. 22 421-477

SEQUENCE LISTING

(1)		GENERAL INFORMATION	
	(i)	APPLICANTS:	
		(A) NAME:	Advanced Technologies (Cambridge) Limited
		(B) STREET:	Millbank, Knowle Green
		(C) CITY:	Staines
		(D) STATE:	Middlesex
		(E) COUNTRY:	England
		(F) POSTAL CODE:	TW18 1DY
	(ii)	TITLE OF INVENTION:	Modification of Plant Fibres
	(iii)	NUMBER OF SEQUENCES:	6
	(iv)	CORRESPONDENCE ADDRESS:	
		(A) ADDRESSEE:	British-American Tobacco Company Limited
		(B) STREET:	Regent's Park Road
		(C) CITY:	Southampton
		(D) STATE:	Hampshire
		(E) COUNTRY:	England
		(F) POSTAL CODE:	SO15 8TL
	(v)	COMPUTER READABLE FORM:	
		(A) MEDIUM TYPE:	Diskette 3.50 inch
		(B) COMPUTER:	Viglen P5/75
		(C) OPERATING SYSTEM:	MS-DOS Windows 95
		(D) SOFTWARE:	Microsoft Word 97
	(vi)	CURRENT APPLICATION DATA:	
		(A) APPLICATION NUMBER:	Not yet known
		(C) CLASSIFICATION:	Not yet known
	(viii)	ATTORNEY/AGENT INFORMATION:	
		(A) NAME:	Mrs. M.R. Walford/ Mr. K.J.H. MacLean
		(C) REFERENCE:	RD-ATC-19
	(ix)	TELECOMMUNICATION INFORMATION:	
		(A) TELEPHONE:	01703 777155
		(B) TELEFAX:	01703 779856

INFORMATION FOR SEQUENCE ID. NO: 1

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	488 base pairs
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	double
(D)	TOPOLOGY	linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Eucalyptus grandis

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 1

```
ATGGGGGGGG CTTGTGGGTA TGGCAACCTG TACAGCCAAG GCTATGGCAC   50
CAACACTGCA GCTTTGAGCA CTGCCCTGTT CAACAATGGC CTGAGCTGCG  100
GGGCATGTTA CGAGATGCGG TGCAACGACG ACCCCAGGTG GTGCCTCCCG  150
GGGACCATCA TGGTCACGGC AACCAACTTT TGCCCTCCCA ACTTGGCCCT  200
CTCCAACGAC AATTGCGGCT GGTGCAACCC CCCTCTCCAG CACTTCGATA  250
TGGCCGAGCC TGCTTTCTTG CAGATTGCCC AGTACAAAGC TGGGATTGTC  300
CAGGTTTCCT TCAGAAGGGT TCCGTGTGTG AAGAAAGGAG GGGTAAGGTT  350
CACCATCAAT GGGCACTCCT ACTTCAACTT GGTGCTGATC ACCAACGTGG  400
GAGGTGCTGG TGATGTCCAT TCCGTTTCCA TCAAGGGCTC GAGGACTGGT  450
TGGCAAGCCA TGTCAAGGAA CTGGGGCAAA AACTGGCA               488
```

INFORMATION FOR SEQUENCE ID. NO: 2

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 475 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: double
  (D) TOPOLOGY linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
  (A) ORGANISM: Eucalyptus grandis

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 2

```
ATGGGGGGGG CATGCGGGTA TGGCAACCTG TACAGCCAAG GCTATGGCAC    50
CAACACTGCA GCTTTGAGCA CTGCCCTGTT CAACAATGGC CTGAGCTGCG   100
GGGCATGTTA CGAGATGCGG TGCAACGACG ACCCCAGGTG GTGCCTCCCG   150
GGGACCATCA TGGTCACGGC AACCAACTTT TGCCCTCCCA ACTTGGCCCT   200
CTCCAACGAC AATGGCGGCT GGTGCAACCC CCCTCTCCAG CACTTCGATA   250
TGGCCGAGCC TGCTTTCTTG CAGATTGCCC AGTACAAAGC TGGGATTGTC   300
CCGGTTTCCT TCAGAAGGGT TCCGTGTGTG AAGAAAGGAG GGGTAAGGTT   350
CACCATCAAT GGGCACTCCT ACTTCAGCTG TGGTGCTGAT CACCAACGTG   400
GGAGGTGCTG GTGATGTCCA TTCCGTTTCC ATCAAGAGCT CGAGGACTGG   450
TTGGCAAGCC ATGTCAAGGA ATTGA                              475
```

INFORMATION FOR SEQUENCE ID. NO: 3

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 494 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
- (A) ORGANISM: Eucalyptus grandis

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 3

```
ATGGGGGGGG CATGTGGTTA CGGGGACCTT CACAGGGCCA CCTATGGCAA    50
GTACAGTGCC GGCTTGAGCT CGATGCTGTT CAACAGAGGG AGTACCTGCG   100
GGGCTTGCTT CGAGCTCCGG TGCGTCGACC ACATTTTGTG GTGCCTCCCT   150
GGTAGCCCGT CGGTGATCCT CACCGCCACC GACTTCTGCC CTCCGAACTA   200
CGGGCTCGCG GCAGATTACG GCGGGTGGTG CAACTTCCCG CAGGAGCACT   250
TCGAGATGTC GGAGGCGGCC TTCGCCGAGA TTGCGGTGCG AAGGGCTGAT   300
GTGGTGCCTA TCCAGTACAG GAGGGTGAAC TGTCTGAGAA GCGGTGGTCT   350
GAGATTCACA TTGAGCGGAA ACTCTCACTT CTTTCAGGTC TTGGTGACGA   400
ATGTAGGCCT AGATGGGGAG GTGATTGCCA TGAAAATGAA GGGATCGAAA   450
ACAGGGTGGA TACCGATGGC AAGAAACTGG GGCAAAAACT GGCA         494
```

INFORMATION FOR SEQUENCE ID. NO: 4

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	437 base pairs
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	double
(D)	TOPOLOGY	linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Eucalyptus grandis

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 4

```
ATGGGTTGCC ACCGGGTCCT TGATCCTTTG ATGGCCACGG AGTGCACATC   50
CCCTGCTCCG CCGACATTGG TTATGAGCAC GAGGTTGAAA TAAGAATGGC  100
CGTTGACGGT GAACCGGATC CCTCCGCTTC TCCTGCACCT CACTCTTCGG  150
TAGGCCACCG GGACGATCCC GGCCCTGTAC TGCGCAATGT GCTGGAAGAC  200
CGGCTGGGAG AGGTCGAAAT GGAGTTGAGG AGGGTCGCAC CACCCTCCTG  250
GAGGGCAGAA GTTGGTCGCC GTGACCACAA TGGCGCCCGG GAGGCACCAC  300
TGCGGGTCGT TCACGCACCG GAGCTCAAAG CACGCGCCGC AGCTCAGCCC  350
ATTGTTGAAC AATGCAGTGC TCAGTGCAGC TGTGTTTGTG CCGTACCCTT  400
GGCTGTATAG ATTCCCATAA CCACACGCCC CCCCCAT                437
```

INFORMATION FOR SEQUENCE ID. NO: 5

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	437 base pairs
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	double
(D)	TOPOLOGY	linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Eucalyptus grandis

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 5

```
ATGGGTTGCC ACCGGGTCCT TGATCCTTTG ATGGCCACGG AGTGCACATC   50
CCCTGCTCCG CCGACATTGG TTATGAGCAC GAGGTTGAAA TAAGAATGGC  100
CGTTGACGGT GAACCGGATC CCTCCGCTTC TCCTGCACCT CACTCTTCGG  150
TAGGCCACAG GGACGATCCC GGCCCTGTAC TGCGCAATGT GCTGGAAGAC  200
AGGCTGGGAG AGGTCGAAAT GGAGTTGAGG AGGGTCGCAC CACCCTCCTG  250
GAGGGCAGAA GTTGGTCGCC GTGACAACAA TGGCGCCCGG GAGGCACCAC  300
TGCGGGTCGT TCACGCACCG GAGCTCAAAG CACGCGCCGC AGCTCAGCCC  350
ATTGTTGAAC AATGCAGTGC TCAGTGCAGC TGTGTTTGTG CCGTACCCTT  400
GGCTGTATAG ATTCCCATAA CCACACGCCC CCCCCAT                437
```

INFORMATION FOR SEQUENCE ID. NO: 6

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 448 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: double
  (D) TOPOLOGY linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
  (A) ORGANISM: Eucalyptus grandis

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 6

```
CCTTGACATG GTCTGCCACC TTGTCCGCGA ACCCTTCACG GCGACCGAGT   50
TGACGTTGCC TGCGCCGCCG ACGTTTGTGA CGAGGACGAG CTTGAAGTAT  100
GAGTTGCCGT TGATGGTGAA CCGGATGCCT CCTCTCCTCC TGCACGTCAC  150
CCTCCTGTAC GCAACGTGGA CGATGCCGGC TCGGTACTTG GCAATGTGCT  200
GGAAGACGGG CTGGGAGATG TCGAAGTGGT GTTGGGGCGG GTTGCACCAT  250
CCGCCGGCGT TGTTTGGGAG GGCGTTGTTT GGCGGGCAGA AGTTTGTGGC  300
GGTGACGACG ATGGAGCCGC CCAGGCACCA CTTTCCGTCG TTCACGCACC  350
GGATCTCGAA GCACGACCCC CAGCTCAGCC CGTTTTTTAA CAGCGCCGTG  400
CTCAGCGCCG CCGTGTTCGT ACCGTAGCCC TGGCTGTACA GGTTGCCG    448
```

INFORMATION FOR SEQUENCE ID. NO: 7

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	19 nucleotides
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY	linear

(ii) MOLECULE TYPE: Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 7

```
ATGGIGGIGC NTGTGGNTA                                   19
```

Key I = Inosine
N = A, G, T or C

INFORMATION FOR SEQUENCE ID. NO: 8

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	20 nucleotides
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY	linear

(ii) MOLECULE TYPE: Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 6

TGCCARTTYT GNCCCCARTT 20

Key R = A or G
Y = T or C
N = A, G, T or C

Figure ~~22~~ 1 **Diagram of Constructs prepared for Plant Transformation:**

C58/pDE, C58/pATC and EHA105/pATC.